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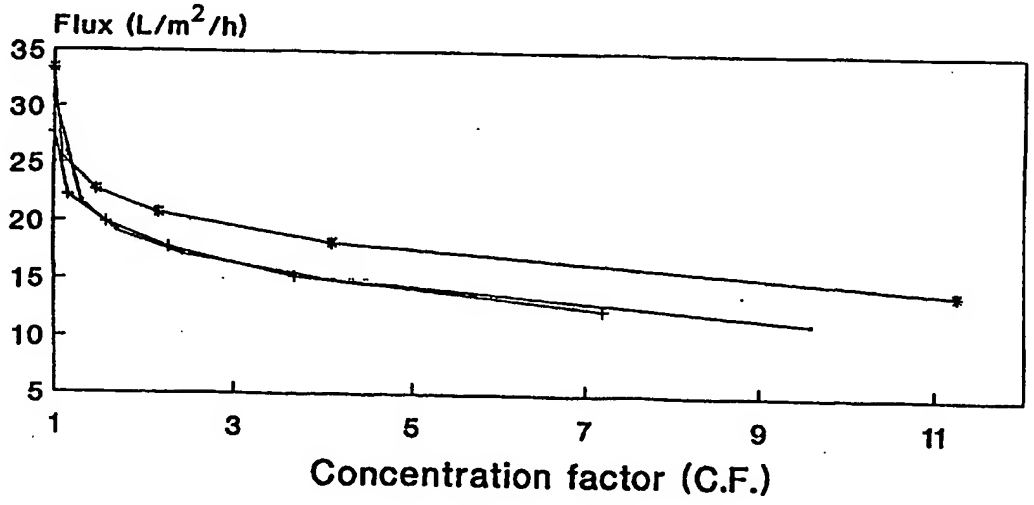

(57) Abstract

The present invention relates to a method for purification of a protein obtained from an aqueous protein solution comprising (a) treatment of the protein solution with a soluble Fe and/or Al compound at a pH between 4 and 9; (b) treatment of the protein solution with one or more flocculating agent(s); (c) clarification of the protein solution; and (d) membrane concentration of said protein solution.

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Al/Fe-Treatment of a Protein Solution, Followed by Membrane Concentration

TECHNICAL FIELD

The present invention relates to a simple and effective method for purification of a protein, in particular an enzyme, obtained from a protein solution, e.g., a fermentation broth.

BACKGROUND ART

Use of Al-compounds for precipitation of impurities from fermentation broths prior to evaporation and amorphous salt precipitation is known (for reference see WO 94/01537 and US Patent 3,795,586). However, vacuum evaporation is from an energy consumption view expensive. Further, only a small concentration factor is possible in the evaporation process due to a high salt and a low molecular weight component level in a fermented solution. An amorphous salt precipitation with, e.g., Na_2SO_4 or $(NH_4)_2SO_4$, is highly salt consuming and the process is not an efficient unit operation for protein purification.

Membrane concentration, e.g., UF concentration, is widely used for protein concentration as the process by leaching out salts and small components potentially should give a more efficient concentration than what is possible when using a vacuum evaporation. However, the membrane filtration is often hampered capacity wise, why large seize equipment and thereby high energy consumption is necessary, and further often hampered efficiency wise allowing only for a limited concentration factor. It is therefore a desideratum for the art to develop procedures to overcome the above mentioned problems.

SUMMARY OF THE INVENTION

It has surprisingly been found that a fermentation broth which has been treated with a soluble Fe and/or Al compound is particularly well suited for membrane concentration resulting in a significant capacity gain.

The present invention provides a method for purification of a protein obtained from an aqueous protein solution comprising

(a) treatment of the protein solution with a soluble Fe and/or Al compound at a pH between 4 and 9;

(b) treatment of the protein solution with one or more flocculating agent(s);

(c) clarification of the protein solution; and

(d) membrane concentration of said protein solution.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which

Fig. 1 shows an ultrafiltration performance of a protease solution at three different aluminate dosings (▪: 0.8 kg $NaAlO_2$ per 100 kg fermentation broth; +: 1.6 kg $NaAlO_2$ per 100 kg fermentation broth; *: 2.4 kg $NaAlO_2$ per 100 kg fermentation broth), the test performed as described in Example 1.

Fig. 2 shows an ultrafiltration performance of an amylase solution at three different aluminate dosings (▪: 0 kg $NaAlO_2$ per 100 kg fermentation broth; +: 1 kg $NaAlO_2$ per 100 kg fermentation broth; *: 4.2 kg $NaAlO_2$ per 100 kg fermentation broth), the test performed as described in Example 2.

Fig. 3 shows an ultrafiltration performance of a protease solution with/without a previous aluminium sulphate treatment (♦: 0.7 kg 30% $Al_2(SO_4)_3 \times 18H_2O$ per 1.54 kg fermentation broth; □: 0 kg $Al_2(SO_4)_3 \times 18H_2O$ per 1.54 kg fermentation broth); the test performed as described in Example 3.

Fig. 4 shows an ultrafiltration performance of a protease solution with/without a previous iron sulphate treatment □: 0.177 kg 30% $Fe_2(SO_4)_3x7H_2O$ per 1.54 kg fermentation broth; ◇: 0 kg $Fe_2(SO_4)_3x7H_2O$ per 1.54 kg fermentation broth); the test performed as described in Example 4.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides a method for purification of a protein obtained from an aqueous protein solution comprising

(a) treatment of the protein solution with a soluble Fe and/or Al compound at a pH between 4 and 9;

(b) treatment of the protein solution with one or more flocculating agent(s);

(c) clarification of the protein solution; and

(d) membrane concentration of said protein solution.

The method of the invention can be applied to purification of a protein obtained from an aqueous protein solution, in particular to purification of a protein such as an enzyme from a fermentation broth.

In a preferred embodiment, the method is applied to purification of proteases, lipases, amylases, cellulases. and oxidoreductases.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically or genetically modified mutants are included. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium

protease described in WO 89/06270.

Preferred commercially available protease enzymes include those sold under the tradenames Alcalase, Savinase, Primase, Durazym, and Esperase by Novo Nordisk A/S (Denmark), those sold under the tradename Maxatase, Maxacal, Maxapem and Properase by Gist-Brocades, those sold under the tradename Purafect and Purafect OXP by Genencor International, and those sold under the tradename Opticlean and Optimase by Solvay Enzymes.

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included.

Examples of useful lipases include a *Humicola lanuginosa* lipase, e.g., as described in EP 258 068 and EP 305 216, a *Rhizomucor miehei* lipase, e.g., as described in EP 238 023, a *Candida* lipase, such as a *C. antarctica* lipase, e.g., the *C. antarctica* lipase A or B described in EP 214 761, a *Pseudomonas* lipase such as a *P. alcaligenes* and *P. pseudoalcaligenes* lipase, e.g., as described in EP 218 272, a *P. cepacia* lipase, e.g., as described in EP 331 376, a P. stutzeri lipase, e.g., as disclosed in BP 1,372,034, a P. fluorescens lipase, a *Bacillus* lipase, e.g., a *B. subtilis* lipase (Dartois et al., (1993), Biochemica et Biophysica acta 1131, 253-260), a *B. stearothermophilus* lipase (JP 64/744992) and a *B. pumilus* lipase (WO 91/16422).

Furthermore, a number of cloned lipases may be useful, including the *Penicillium camembertii* lipase described by Yamaguchi et al., (1991), Gene 103, 61-67), the *Geotricum candidum* lipase (Schimada, Y. et al., (1989), J. Biochem., 106, 383-388), and various *Rhizopus* lipases such as a *R. delemar* lipase (Hass, M.J et al., (1991), Gene 109, 117-113), a *R. niveus* lipase (Kugimiya et al., (1992), Biosci. Biotech. Biochem. 56, 716-719) and a *R. oryzae* lipase.

Other types of lipolytic enzymes such as cutinases may also be useful, e.g., a cutinase derived from *Pseudomonas mendocina* as described in WO 88/09367, or a cutinase derived from *Fusarium solani pisi* (e.g. described in WO 90/09446).

Especially suitable lipases are such as M1 Lipase™, Luma fast™ and Lipomax™ (Gist-Brocades), Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S), and Lipase P "Amano" (Amano Pharmaceutical Co. Ltd.).

Amylases: Suitable amylases (α or ß) include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Amylases include, for example, a-amylases obtained from a special strain of B. licheniformis, described in more detail in British Patent Specification No. 1,296,839. Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (available from Novo Nordisk A/S) and Rapidase™ and Maxamyl P™ (available from Gist-Brocades).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Suitable cellulases are disclosed in US 4,435,307, which discloses fungal cellulases produced from Humicola insolens. Especially suitable cellulases are the cellulases having colour care benefits. Examples of such cellulases are cellulases described in European patent application No. 91202879.2, filed November 6, 1991 (Novo Nordisk A/S).

Commercially available cellulases are Celluzyme™ produced by a strain of Humicola insolens, (Novo Nordisk A/S), and KAC-500(B)™ (Kao Corporation).

Oxidoreductases: Oxidoreductases are defined and described in, e.g., "Enzyme Nomenclature 1992, Academic press, Inc., San Diego". They belong to a class of enzymes that catalyses transfer of electrons from one substance to another (oxidation-reduction). Oxidoreductases include dehydrogenases, reductases, oxidases, transhydrogenases, catalases, peroxidases, and oxygenases.

More specific examples include horseradish peroxidase, ligninases and other peroxidases, and oxidases such as laccases.

Examples of microorganism genera which may be used for production of suitable oxidoreductases are: *Trametes*,

Rhizoctonia, *Pseudomonas*, *Bacillus*, *Streptomyces*, *Hygrophorus*, *Coprinus*, *Polyporus*, *Candida*, *Curvularia*, *Cercospora*, *Myceliophtora*, *Aspergillus*, and *Scytalidium*. The invention, however, is not restricted to enzymes derived from the above mentioned taxa. All microorganisms producing oxidoreductases with the desired properties may be used in relation to this invention.

The oxidoreductase is preferably a laccase (EC 1.10.3.2), a catalase (EC 1.11.1.6), a peroxidase (EC 1.11.1.7), or an oxidase.

A suitable peroxidase is one producible by a microorganism such as a fungus or a bacterium. In a preferred embodiment, the peroxidase is derived from *Coprinus*, e.g., *C. cinereus* or *C. macrorhizus*, or from *Bacillus*, e.g., *B. pumilus*, particularly a peroxidase according to International Patent Application WO 91/05858.

The enzyme may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said enzyme as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the enzyme in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a *Coprinus* sp., in particular *C. macrorhizus* or *C. cinereus* according to WO 92/16634.

Catalases are known both from animal sources (e.g. cow liver) and from many different microorganisms. JP Patent Application 2-76579 discloses catalase from *Aspergillus niger* strain NFAG-2. GB Patent No. 2,216,149 discloses catalase from *Penicillium*. In a preferred embodiment of the invention the catalase is obtained from strains of *Scytalidium* and *Humicola* as described in WO 92/17571.

The method of the invention may be applied to an untreated fermentation broth or to a fermentation broth that has first been subjected to, e.g., a pH adjustment, a tempera-

ture adjustment, a water dilution and/or one or more solid/liquid separatory techniques such as flocculation, centrifugation, filtration or micro filtration.

According to the invention it has been found that an addition of a soluble Fe and/or Al compound to a protein solution results in a liquid which is surprisingly well fitted for membrane concentration as the flux as well as the purity of the protein is increased.

Addition of a soluble Fe and/or Al compound to a fermentation broth will give an insoluble precipitate mainly consisting of Al and/or Fe hydroxide and impurities such as carbohydrates if the pH of the fermentation broth is between 4 and 9. It is important that the pH of the fermentation broth is kept between pH 4 and pH 9. Normally the precipitation process proceeds in the most efficient manner at lower pH-values. However, many proteins such as most enzymes exhibit low stability at low pH-values. Thus, a compromise pH-value with a reasonably efficient process and a reasonably good protein stability has to be chosen.

It is to be understood that the addition of a soluble Al/Fe compound to the fermentation broth with subsequent precipitation of the hydroxide will change the pH, but pH should be readjusted to pH 4 - 9 all through the precipitation phase and a sufficient time after until the pH of the solution is stabilized within the pH range 4-9.

It may, in some cases, be an advantage to use a mixture of acidic and alkaline Al/Fe-compounds in order to minimize the amount of acid/base needed for the pH adjustment, preferably a pH adjustment is not necessary at all. An example of such a combination of an acidic salt and an alkaline salt is sodium aluminate and aluminium sulphate.

If a pH adjustment is necessary any acid or base may be used, but formic acid or acetic acid are preferred as acids.

The minimum amount of an Al/Fe compound which will precipitate no more than a negligible amount of impurities is dependent upon the kind and concentration of the impurities

but the minimum amount will typically be 0.02 moles Al and/or Fe per liter of protein solution, preferably 0.04 moles Al and/or Fe per liter of protein solution.

The maximum amount of an Al/Fe compound which will precipitate no more than a negligible amount of protein is dependent upon the kind and concentration of the protein, but the maximum amount will typically be around 1.2 moles Al and/or Fe per liter of protein solution, preferably 1.0 moles Al and/or Fe per liter of protein solution.

According to the invention any soluble Fe or Al compound or any mixture thereof may be used, in particular $Al_2(SO_4)_3$, $NaAlO_2$, $K_2Al_2O_4$, $Al(NO_3)_3$, $AlCl_3$, Al-acetate, Al-formate, polymer aluminiumhydroxychloride (e.g., EKOFLOCK available from Boliden), $Fe_2(SO_4)_3$, Fe(III)-formate, Fe(III)-acetate, Fe(II)-formate and Fe(II)-acetate.

In WO 94/01537 it is disclosed that in a preferred embodiment the mixture comprising soluble aluminate also contains a water miscible solvent such as an alcohol. We have found that an optimal precipitation is facilitated by adding one or more flocculating agents.

According to the invention useful flocculating agents are salts such as Ca- and Mg-salts, in particular phosphates, sulphates or chlorides, e.g., calcium chloride, and inorganic and/or organic polymers which may be cationic, anionic or nonionic, in particular a combination of cationic and anionic polymers.

Before a membrane concentration of the protein solution, e.g., the fermentation broth, can take place the Al/Fe precipitate should be removed by one or more clarification processes which will typically be a centrifugation, a filtration, a microfiltration or a combination thereof. The Al/Fe precipitate may conveniently be removed by drumfiltration followed by a filter press filtration as described in Examples 1 and 2.

According to the invention the membrane concentration may be performed using any membrane equipment known in the art, but it is preferred that the membrane concentration is

done using ultrafiltration techniques such as hollow fiber, spiral rounds or plate and frame units. The membranes may be made of a variety of materials such as polysulfone membranes. The preferred cut off value will depend on the properties of the protein in question but usually a cut off value in the interval of from 1 to 100 kD is preferred.

The protein concentrate achieved according to the invention may be further purified in a variety of ways such as by using chromatographic methods, adsorption and/or crystallization processes.

The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

The following example shows the unique correlation between the use of aluminate and the improvement of capacity (flux) in the following ultrafiltration on polysulfone membrane when purificating a protease from a fermentation broth.

A fermentation broth containing protease (Savinase™) from Bacillus lentus, obtained as described in US Patent No. 3,723,250, was subjected to the method of the invention.

Three tests were performed with three different levels of aluminate in the following way:

After fermentation water and calcium chloride was added to the protease containing broth in the amounts given in Table 1. Then sodium aluminate (dosed as a 20% w/v solution) was added (0.8 kg per 100 kg broth in test A; 1.6 kg per 100 kg broth in test B and 2.4 kg per 100 kg broth in test C) while at the same time pH of the solution was kept at pH 8 by adding formic acid. Lastly the flocculating agents (Superfloc C 521 and Superfloc A 130) were added in the amounts given in Table 1:

Table 1

Chemicals	Test A	Test B	Test C
Broth	100 kg	100 kg	100 kg
Water	219 kg	218 kg	213 kg
$CaCl_2x2H_2O$	2 kg	2 kg	2 kg
$NaAlO_2$ (53-55% Al_2O_3)	0.8 kg	1.6 kg	2.4 kg
20% Superfloc C 521	2 kg	2 kg	2. kg
0.1% Superfloc A 130	20 kg	20 kg	25 kg

Hereafter the so treated fermentation broth was drum-filtered and further filtered on a filter press. Finally the solution was ultrafiltered at 7-15°C on a 18 m^2 DDS 35 L plate and frame system with GR 61 PP membranes. The membranes were made from polysulfone with a cut off value of approx. 20,000 D.

The enzyme concentration of the drum filtrate in test A, B and C was at the same level.

The flux characteristics of the three tests are shown in Figure 1 (▪: 0.8 kg $NaAlO_2$ per 100 kg fermentation broth (test A); +: 1.6 kg $NaAlO_2$ per 100 kg fermentation broth (test B); *: 2.4 kg $NaAlO_2$ per 100 kg fermentation broth (test C)). Concentration factor (C.F.) was calculated as $V_{initial}/V_{concentrate}$. Relevant flux data are shown in Table 2.

Table 2

	Test A	Test B	Test C
Average Flux, $L/m^2/h$ (C.F: 1-7)	19.1	19.2	22.4
Flux increase:	0%	≈0%	17%

Relevant purity data on the concentrated products (A,

B and C) are shown in Table 3.

Table 3

Data	Test A	Test B	Test C
Activity/g dry matter (RI) (non permeable dry matter)	100%	114%	131%
OD_{440nm} per activity unit	100%	67%	52%

The purity data shown in Table 3 clearly indicate that a higher dose of aluminate gives a higher purity of the enzyme per g dry matter and lower concentration of coloured species per activity unit.

EXAMPLE 2

The following example shows the unique correlation between the use of aluminate and the improvement of capacity (flux) in the following ultrafiltration on hollow fiber module when purifying an amylase from a fermentation broth.

A fermentation broth containing a Bacillus licheniformis amylase (Termamyl™) was subjected to the method of the invention.

Three tests were performed with different levels of aluminate in the following way:

After fermentation water and calcium chloride were added to the amylase containing broth in the amounts given in Table 4. Then sodium aluminate (dosed as a 20% w/v solution) was added (0 kg per 100 kg broth in test A; 1 kg per 100 kg broth in test B and 4.2 kg per 100 kg broth in test C) while at the same time pH of the solution was kept at pH 8 by adding formic acid. Lastly the flocculating agents (Superfloc C 521 and Superfloc A 130) were added in the amounts given in Table 4:

Table 4

Chemicals	Test A	Test B	Test C
Broth	100 kg	100 kg	100 kg
Water	216 kg	213 kg	185 kg
$CaCl_2x2H_2O$	1 kg	0.5 kg	1 kg
$NaAlO_2$ (53-55% Al_2O_3)	0 kg	1 kg	4.2 kg
20% Superfloc C 521	6.6 kg	6.6 kg	3.5 kg
0.1% Superfloc A 130	6.5 kg	10 kg	40 kg

The water level was adjusted to give the same enzyme concentration in all of the three flocculated suspensions.

The flocs were separated from the clear liquid by drum filtration without any additional dilution with tab water.

After the drumfiltration the product was further filtered on a filter press to give a NTU value below 100 and finally ultrafiltered at 7-15°C on a Romicon/Koch 2.3 m^2 hollow fibre module (PM5, 1.1 mm in ID) with a cut off value of 5000 D. The module was operated at a TMP value of approx. 1.2 Bar and a cross flow velocity of approx. 1 m/s. After 10 hours of operation the ultrafiltration was stopped.

In Figure 2 the flux (L/m^2/hour) as a function of the concentration factor is shown for the three different types of filtrates (▪: 0 kg $NaAlO_2$ per 100 kg fermentation broth (test A); +: 1 kg $NaAlO_2$ per 100 kg fermentation broth (test B); *: 4.2 kg $NaAlO_2$ per 100 kg fermentation broth (test C)).

In Table 5, the flux results are summarized.

Table 5

	Test A	Test B	Test C
Average Flux, $L/m^2/h$ (C.F.: 1-1.8)	8.4	9.2	10.8
Flux increase:	0%	10%	29%

Relevant purity data on the concentrated products are shown in Table 6.

Table 6

Data	Test A	Test B	Test C
Activity/g dry matter (RI) (non permeable dry matter)	100%	104%	116%
OD_{440nm} per activity unit	100%	73%	27%
g Carbohydrate per activity unit	100%	100%	70%

The purity data shown in Table 6 clearly indicate that a higher dose of aluminate gives lower carbohydrate levels and a low concentration of coloured species.

EXAMPLE 3

The following example shows the unique correlation between the use of aluminium sulphate (natural alunogenite) and the improvement of capacity (flux) in the following ultrafiltration on polysulphone membrane when purificating a protease from a fermentation broth.

A fermentation broth containing protease (Savinase™) from Bacillus lentus, obtained as described in US Patent No. 3,723,250 was subjected to the method of the invention.

Two tests were performed. The first test (Test A) without aluminium sulphate and the second test (Test B) with 14% w/w $Al_2(SO_4)_3x18H_2O$. Both at pH=8.0.

After fermentation water and calcium chloride were added to the protease containing broth in the amounts given in Table 7. Then aluminium sulphate (dosed as a 30% w/w solution) was added (Test B) while at the same time pH of the solution was kept at pH=8 by adding sodium hydroxide. Lastly the flocculating agents (Superfloc C521 and Superfloc A130) were added in the amounts given in Table 7.

Table 7

Chemicals	Test A	Test B
Broth	1.54 kg	1.54 kg
Water	3.0 kg	1.85 kg
$CaCl_2x2H_2O$	0.078 kg	0.078 kg
30 % $Al_2(SO_4)_3x18H_2O$	0 kg	0.70 kg
Sodium hydroxide	0.014 kg	0.559 kg
20 % Superfloc C521	0.028 kg	0.028 kg
0.1% Superfloc A130	0.42 kg	0.42 kg

Hereafter the so treated fermentation broths were filtered on a pressure nutsch.

Finally the solutions from Test A and Test B were ultrafiltered at 10°C on 27 cm^2 Amicon cells with PM10 membranes at 3.0 Bar. The membranes were made of polysulphone with a cut off value of approx. 10.000 D.

The enzyme concentration of the filtrates in the two tests were of the same level.

The flux characteristics of the two tests are shown in Figure 3. Concentration factor (C.F.) was calculated as $V_{initial}/V_{concentrate}$, where V is Volume. Yield was calculated as $NPU_{final}/NPU_{initial}*100$, where NPU is the amount of Novo Protease Units. Relevant flux data are shown in Table 8.

The protease units were determined as described below:

Proteolytic Activity

The proteolytic activity may be determined using casein as substrate.

One Casein Protease Unit (NPU) is defined as the amount of enzyme liberating 1 mM of primary amino groups (determined

by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

Table 8

Data	Test A	Test B
Average flux, $L/m^2/h$ (C.F.: 1 - 3)	11.7	15.5
Flux increase (%)	0 %	33 %
Yield (%)	100 %	98 %

Relevant purity data on the concentrated products (A and B) are shown in Table 9.

Table 9

Data	Test A	Test B
Activity per g dry matter (RI)	100 %	110 %
OD_{440nm} per activity unit	100%	34%

The purity data shown in Table 9 clearly indicate that a higher dose of aluminum sulphate gives a higher purity of the enzyme per g dry matter and lower concentration of coloured species per activity unit.

EXAMPLE 4

The following example shows the unique correlation between the use of iron sulphate and the improvement of capacity (flux) in the following ultrafiltration on polysulphone membrane when purifying a protease from a fermentation broth.

A fermentation broth containing protease (Savinase™) from Bacillus lentus, obtained as described in US Patent No. 3,723,250 was subjected to the method of the invention.

Two tests were performed. The first test (Test A) without iron sulphate and the second test (Test B) with 3.45% w/w $Fe_2(SO_4)_3x7H_2O$. Both at pH=8.0.

After fermentation water and calcium chloride were added to the protease containing broth in the amounts given in Table 10. Then iron sulphate (dosed as a 30% w/w solution) was added (Test B) while at the same time pH of the solution was kept at pH=8 by adding sodium hydroxide. Last the flocculating agents (Superfloc C521 and Superfloc A130) were added in the amounts given in Table 10.

Table 10

Chemicals	Test A	Test B
Broth	1.54 kg	1.54 kg
Water	3.11 kg	2.77 kg
$CaCl_2x2H_2O$	0.078 kg	0.078 kg
30 % $Fe_2(SO_4)_3x7H_2O$	0 kg	0.177 kg
Sodium hydroxide	0.02 kg	0.21 kg
20 % Superfloc C521	0.028 kg	0.028 kg
0.1% Superfloc A130	0.42 kg	0.42 kg

Hereafter the so treated fermentation broth was filtered on a pressure nutsch. Finally the solutions from Test A and Test B were ultrafiltered at 10°C and 3 bar(o) on 27 cm^2 Amicon cells with PM10 membranes. The membranes were made of polysulphone with a cut off value of appr. 10.000 D.

The enzyme concentration of the filtrates in the two tests were of the same level.

The flux characteristics of the two tests are shown in Figure 4. Concentration factor (C.F.) was calculated as $V_{initial}/V_{concentrate}$, where V is Volume. Yield was calculated as $NPU_{final}/NPU_{initial}*100$, where NPU is the amount of Novo Protease Units. Relevant flux data are shown in Table 11.

Table 11

Data	Test A	Test B
Average flux, $L/m^2/h$ (C.F.: 1 - 3)	13.3	16.5
C.F.	3.11	3.61
Flux increase (%)	0 %	24 %
Yield (%)	96 %	97 %

Relevant purity data on the concentrated products (A and B) are shown in Table 12.

Table 12

Data	Test A	Test B
Activity per g dry matter (RI)	100 %	104 %
OD_{440nm} per activity unit	100 %	47 %

The purity data shown in Table 12 clearly indicate that a higher dose of iron sulphate gives a higher purity of the product per g dry matter and lower concentration of coloured

species per activity unit.

CLAIMS

1. A method for purification of a protein obtained from an aqueous protein solution comprising
(a) treatment of the protein solution with a soluble Fe and/or Al compound at a pH between 4 and 9;
(b) treatment of the protein solution with one or more flocculating agent(s);
(c) clarification of the protein solution; and
(d) membrane concentration of said protein solution.

2. A method according to claim 1, wherein the protein solution is a fermentation broth.

3. A method according to claim 1, wherein the Al compound is selected from the group consisting of $Al_2(SO_4)_3$, $NaAlO_2$, $K_2Al_2O_4$, $AlCl_3$, $Al(NO_3)_3$, Al-acetate, Al-formate and any mixtures hereof.

4. A method according to claim 1, wherein the Fe compound is selected from the group consisting of $Fe_2(SO_4)_3$, Fe(III)-formate, Fe(III)-acetate, Fe(II)-formate, Fe(II)-acetate and any mixtures hereof.

5. A method according to any preceding claim, wherein the Al and/or Fe compound is added at a concentration of 0.02-1.2 moles per liter of protein solution, preferably at a concentration of 0.04-1.0 moles per liter of protein solution.

6. A method according to any of claims 1-5, wherein the flocculating agents are selected from the group consisting of salts and polymers.

7. A method according to claim 1, wherein the clarification is performed by centrifugation, filtration, microfiltration or a combination thereof.

8. A method according to claim 1, wherein the membrane concentration is ultrafiltration.

9. A method according to claim 1, wherein the protein is an enzyme.

10. A method according claim 9, wherein the enzyme is an oxidoreductase, a protease, a lipase, a cellulase or an amylase.

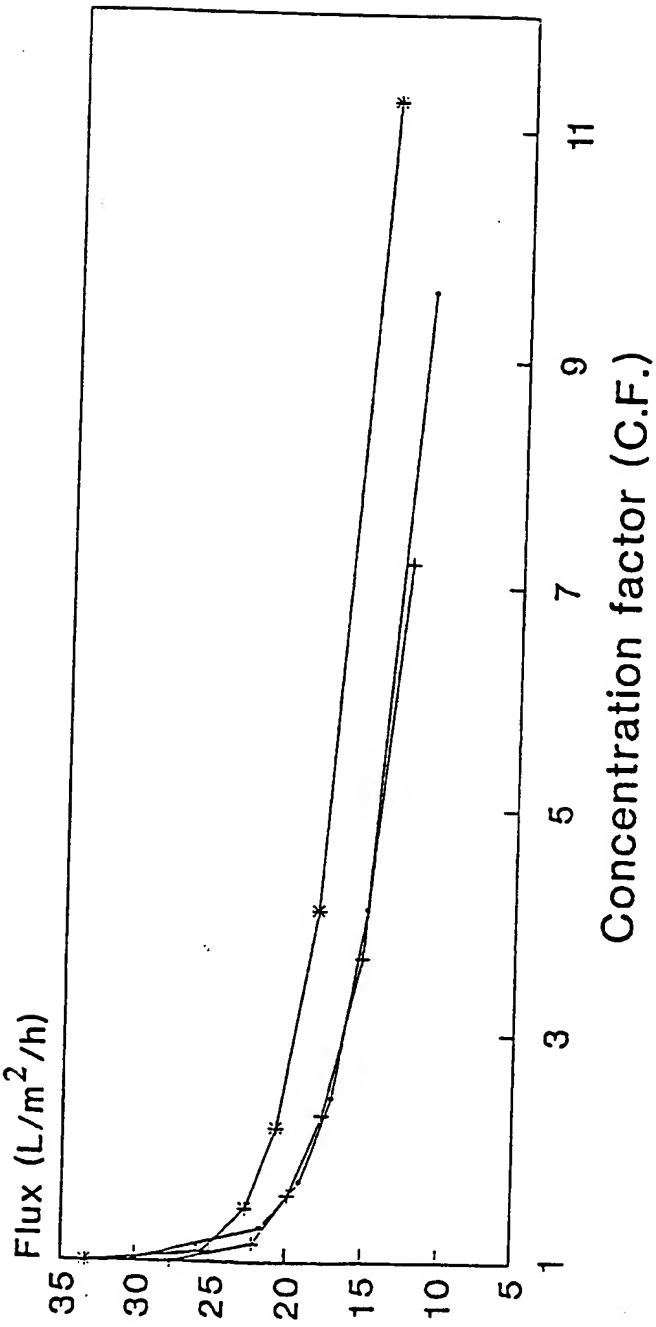

Fig. 1

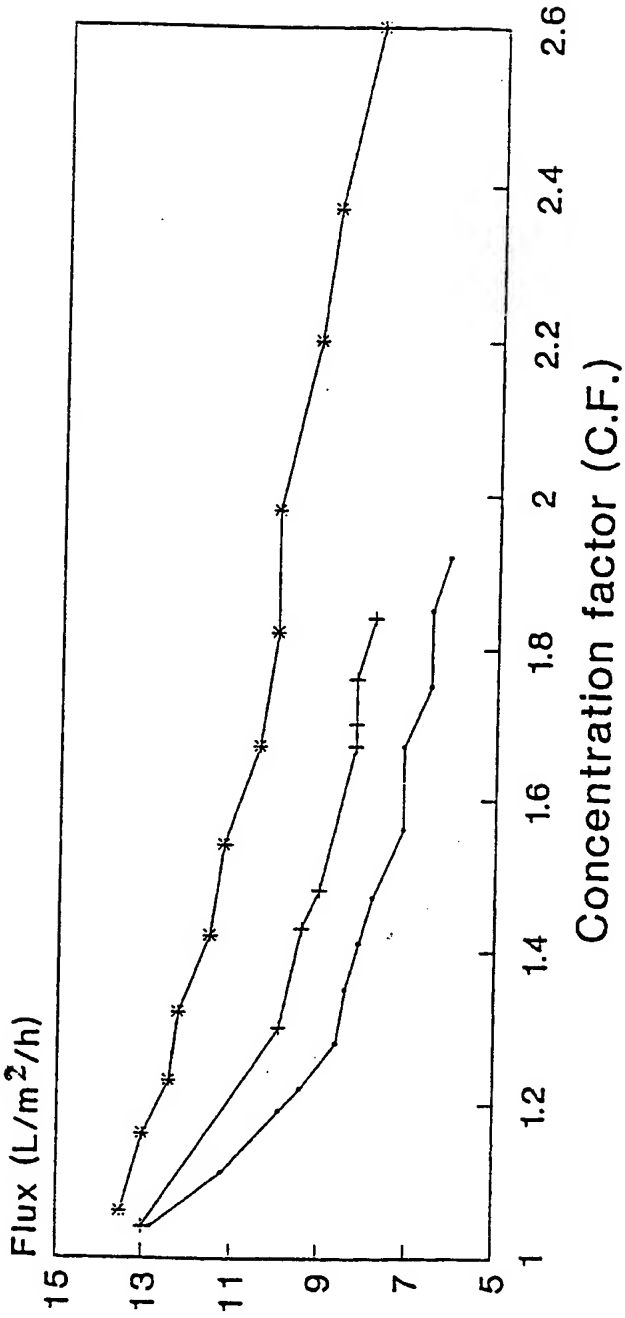
Flux (L/m²/h)
15
13
11
9
7
5
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
Concentration factor (C.F.)

Fig. 2

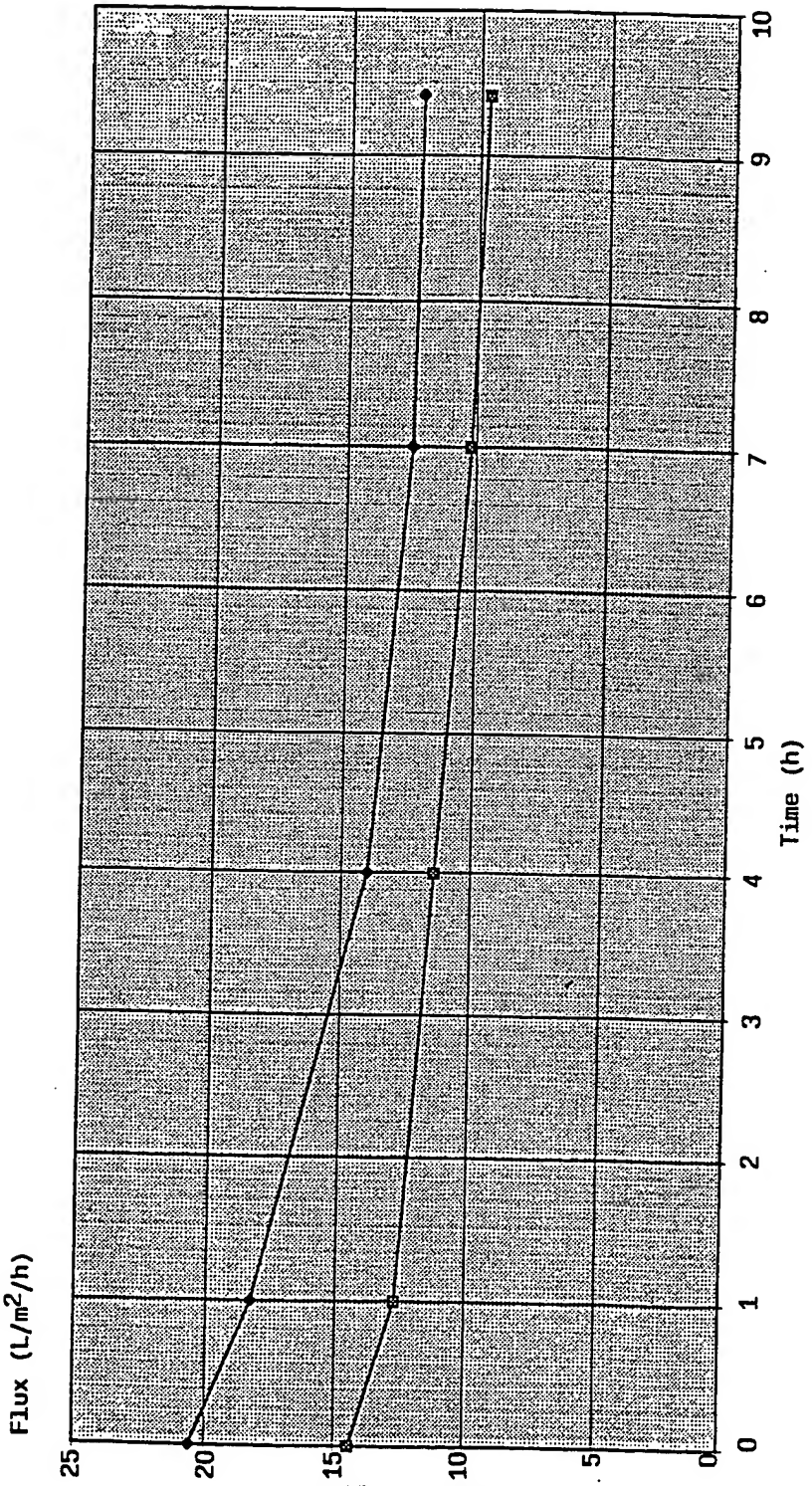

Fig. 3

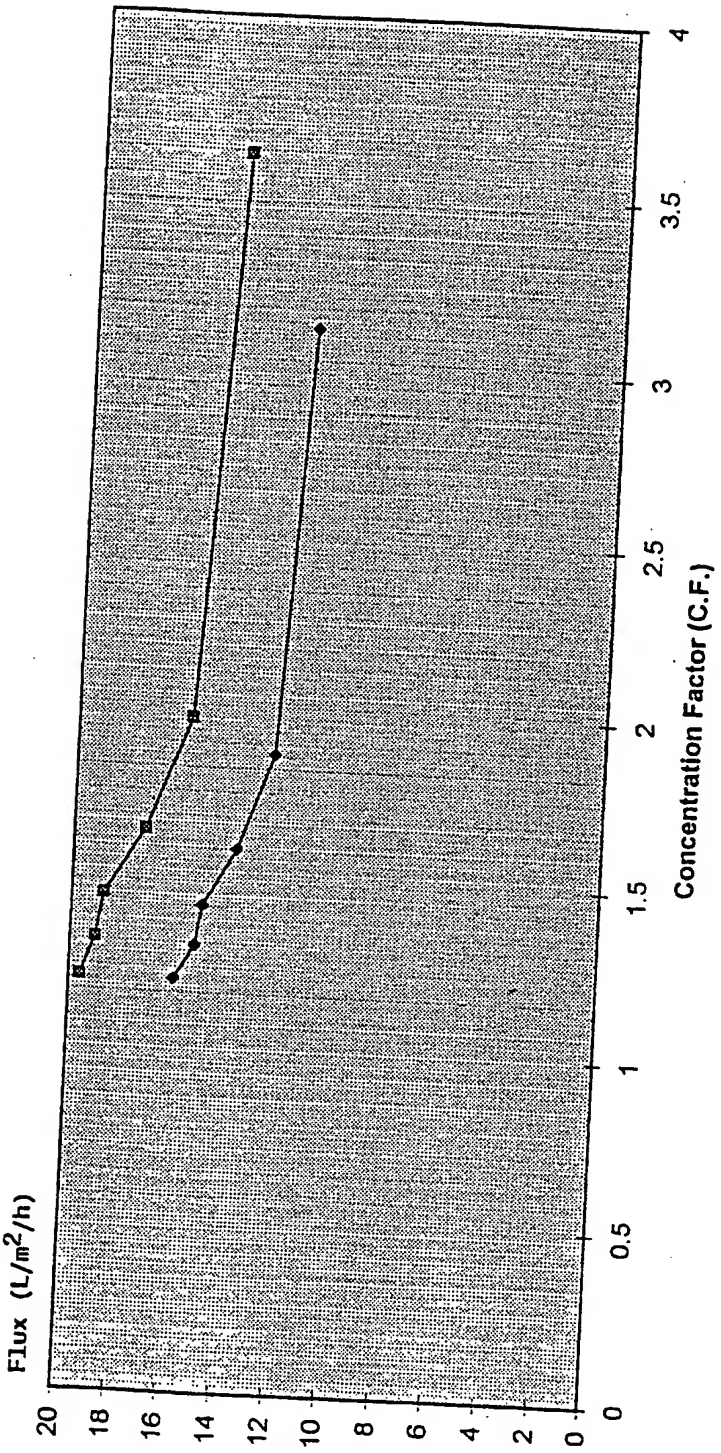
Flux (L/m2/h)
20
18
16
14
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
Concentration Factor (C.F.)

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00229

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/36, C07K 1/30, C12N 9/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPAT, USPM

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9401537 A1 (NOVO NORDISK A/S), 20 January 1994 (20.01.94), see claims ---	1-10
X	US 3795586 A (J. ZIFFER), 5 March 1974 (05.03.74), column 2, line 45 - line 72; column 3, line 1 - line 2; column 3, line 16 - line 20, claims ---	1-10
A	Eur. J. Biochem, Volume 189, 1990, Alexander McPherson, "Current approaches to macromolecular crystallization", page 1 - page 23, see whole document ---	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 1996	30 -08- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Åke Lindberg Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00229

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0277043 A2 (INTERNATIONAL MINERALS AND CHEMICAL CORPORATION), 3 August 1988 (03.08.88), see example 10 and claims	1-10
X	WO 9209687 A1 (HENKEL KOMMANDITGESELLSCHAFT AUF AKTIEN), 11 June 1992 (11.06.92), see claims	1-10
X	WO 9013632 A1 (HENKEL KOMMANDITGESELLSCHAFT AUF AKTIEN), 15 November 1990 (15.11.90), see claims	1-10
A	US 4894444 A (E.M. SCATTERGOOD ET AL), 16 January 1990 (16.01.90), see whole document	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/96

International application No.
PCT/DK 96/00229

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9401537	20/01/94	EP-A- 0651791	10/05/95
		FI-A- 950094	09/01/95
		JP-T- 7508175	14/09/95
US-A- 3795586	05/03/74	CA-A- 947681	21/05/74
		CA-A- 978489	25/11/75
		DE-A- 2039752	25/02/71
		FR-A- 2056864	14/05/71
		NL-A- 7011845	15/02/71
		US-A- 3700561	24/10/72
EP-A2- 0277043	03/08/88	JP-A- 63216898	09/09/88
WO-A1- 9209687	11/06/92	AT-T- 136583	15/04/96
		DE-A- 4037530	27/05/92
		DE-D- 59107671	00/00/00
		EP-A,B- 0559676	15/09/93
		JP-T- 6502763	31/03/94
		US-A- 5453200	26/09/95
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US-A- 4894444	16/01/90	NONE	